

## CAMBIDIUM/XYLEM-PREFERRED PROMOTERS AND USES THEREOF

### RELATED APPLICATION

[0001] This application claims priority of application Serial No. 60/560,227 filed April 6, 2004, and incorporated by reference in its entirety.

### FIELD OF THE INVENTION

[0002] The invention relates generally to the field of molecular biology, biochemistry and agriculture. More particularly, the invention relates to polynucleotides suitable for regulating gene expression in plants and generation of transgenic plants with improved quality and productivity.

### BACKGROUND AND PRIOR ART OF THE INVENTION

[0003] Modification of a plant trait through genetic engineering depends upon the insertion into the plant genome of a polynucleotide construct containing the gene of interest, operably linked to a promoter that is functional in the transgenic plant. Within a plant genome, any single gene is, in general, operably linked to a promoter that will determine when and where, within the plant tissues and organs, the gene should be expressed. Therefore if one wants to express a gene of interest in specific tissues or organs within a transgenic plant and in a temporally regulated manner, tissue-preferred promoters must be used. On the other hand, expression in all plant tissues throughout the plant's life cycle could be achieved by using constitutive promoters.

[0004] In a number of situations the expression of particular genes in particular tissues or organs confers a specific phenotype of interest to the plant. For example, if one wants to improve the nutritional quality of cereal seeds, a gene that confers such phenotype using seed-specific promoters is inserted, rather than using constitutive promoters that would allow the gene to be expressed in all plant tissues causing, in some cases, undesirable phenotypes. In another example, if one wants to increase the amount of cellulose in the developing vascular tissues of a forest tree, one would introduce into the plant genome a xylem- and/or cambium-preferred promoter operably linked to a heterologous gene encoding an enzyme involved in cellulose metabolism such that more cellulose molecules could be produced in the developing plant xylem. In another example, the desired phenotype could be obtained by inhibiting the expression of an

endogenous gene within a specific plant tissue. This could be done by introducing a construct comprising a tissue-preferred promoter operably linked to a polynucleotide that would inhibit the expression of the endogenous gene, either by anti-sense hybridization or by RNA silencing (Matzke (ed.) et al. (2000) *Plant Gene Silencing*. Kluwer Academic Publishers).

[0005] Thus far, the production of genetically engineering plants expressing useful and/or desirable traits requires the availability of promoters that permit the gene or genes of interest to be expressed in a tissue- and timing-specific manner. Thus, isolation and characterization of tissue-preferred, particularly cambium/xylem-preferred, promoters that can serve as regulatory regions for expression of heterologous nucleotide sequences of interest in a tissue-preferred manner is essential for the genetic engineering of plants exhibiting particular traits.

## SUMMARY OF THE INVENTION

[0006] The present invention relates to isolated regulatory nucleic acid molecules from the genome of *Populus* sp, and methods for regulating expression of heterologous nucleotide sequences in plant tissues, such as in a xylem and/or cambium-preferred manner. It is an object of the invention to provide isolated nucleic acid molecules which represent promoters able to direct tissue-specific expression of genes of interest. The regulatory nucleic acid molecules of the present invention correspond to promoter sequences of genes which are preferably expressed in the cambium and/or in the xylem of *Populus* sp. Genes encoding isoforms of sucrose synthase (SuSy), alpha-tubulin (TUB), arabinogalactan protein (ARAB), caffeic acid 3-O-methyltransferase (COMT), cinnamyl alcohol dehydrogenase (CAD), cinnamate 4-hydroxylase (C4H), cinnamoyl CoA reductase (CCR), ferulate-5-hydroxylase (F5H), sinapyl alcohol dehydrogenase (SAD), UDP-D-glucuronate carboxy-lyase (UDP), lipid transfer protein (LTP) and ag-13 (AG13) were found to be expressed in the cambium/xylem tissue of *Populus* sp. and their promoters, which are the subject of the invention, have been isolated, cloned and validated. When these promoters are associated in a transgenic plant with genes other than those to which they were originally linked, the genes in question are preferably expressed in the cambium and/or in the xylem of said transgenic plant. Methods of using the cambium/xylem-preferred promoters disclosed herein, for

regulating expression of heterologous nucleotide sequences in cambium and/or xylem-preferred manner in a plant, are provided.

[0007] The cambium/xylem-preferred promoters were identified through the analysis of a collection of Expressed Sequence Tags (ESTs) from *Populus* sp, representing apical shoot, bark, cambium, seed, xylem, leaf and root tissue. Based on the expression profile of those ESTs among the different tissues, the twelve genes referred to supra were shown to be highly and preferably expressed in the cambium and/or in the xylem of *Populus*.

[0008] The cambium/xylem-preferred promoters of the invention are set forth at SEQ ID NOS.: 1-12. Fragments of these nucleotide sequences, i.e., those set forth in SEQ ID NOS.: 1-12 comprising at least 20 consecutive nucleotides are also a feature of this invention. The smaller fragments, while not necessarily encoding promoters or proteins with promoter activity, can function as antisense molecules and disable naturally occurring and expressed genes. The compositions of the invention further comprise nucleotide sequences having at least 65% identity to the sequences set forth in SEQ ID NOS.: 1-12 or a fragment thereof, and nucleotide sequences that hybridize under high stringency conditions to any one of the aforementioned sequences.

[0009] "Stringent conditions" as used herein, refers to parameters with which the art is familiar, such as hybridization in 3.5xSSC, 1xDenhardt's solution, 25mM sodium phosphate buffer (pH 7.0), 0.5% SDS, and 2mM EDTA for 18 hours at 65°C, followed by 4 washes of the filter at 65°C for 20 minutes, in 2xSSC, 0.1% SDS, and a final wash for up to 20 minutes in 0.5xSSC, 0.1% SDS, or 0.3xSSC and 0.1% SDS for greater stringency, and 0.1xSSC, 0.1% SDS for even greater stringency. Other conditions may be substituted, as long as the degree of stringency is equal to that provided herein, using a 0.5xSSC final wash.

[0010] Other facets of the present invention include constructs, such as expression vectors comprising the promoters operably linked to a nucleotide sequence of interest, which encodes a desired protein. The promoters disclosed herein are capable of driving expression of polynucleotides of interest in a plant cell and said promoters comprise any one of the nucleotide sequences of the present invention.

[0011] Also a part of the invention are recombinant plants or plant cells having stably incorporated into their genomes any one of the constructs described above or the promoter itself.

[0012] Methods of the invention also include methods for stably incorporating the products of the invention into cells.

### **BRIEF DESCRIPTION OF THE DRAWINGS**

[0013] FIG. 1 schematically illustrates the plasmid vector pAPROM-ATG+promoter comprising the GUS reporter gene operably linked to a promoter sequence. Promoters of the invention were cloned in this plasmid vector in substitution of the represented promoter sequence.

[0014] FIG. 2 shows the expression profile in a set of *Populus* tissues of SuSy, TUB, ARAB, UDP, LTP and AG13 genes, which are under the control of the promoters of the invention in *Populus*.

[0015] FIG. 3 shows the expression profile in a set of *Populus* tissues of COMT; CAD, C4H, CCR, F5H and SAD genes, which are under the control of the promoters of the invention in *Populus*.

[0016] FIG. 4 schematically illustrates the plasmid vector pALELLYXgi which is another embodiment of the invention.

[0017] FIGS. 5A and 5B show beta glucuronidase activity in the flowering stem of *Arabidopsis* plants, transformed in accordance with Example 3.

### **DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS**

[0018] The compositions of the present invention comprise novel nucleotide sequences for plant promoters, particularly cambium/xylem-preferred promoters for the *Populus* (woody aspen) genes encoding sucrose synthase (SuSy), alpha-tubulin (TUB); arabinogalactan protein (ARAB), caffeic acid 3-O-methyltransferase (COMT), cinnamyl alcohol dehydrogenase (CAD), cinnamate 4-hydroxylase (C4H), cinnamoyl CoA reductase (CCR), ferulate-5-hydroxylase (F5H), sinapyl alcohol dehydrogenase (SAD), UDP-D-glucuronate carboxy-lyase (UDP), lipid transfer protein (LTP) and ag-13 (AG13). The nucleotide sequences for these promoters are set forth in SEQ ID NOS.: 1-12, respectively. These promoters were isolated from the 5' untranslated region flanking the transcription initiation sites of their respective genes. Methods for

the isolation of the promoters are well known in the art and include bioinformatic tools for gene assembly such as Phred, Phrap, Consed (Gordon *et al.* (1998) *Genome Research.* 8:195-202), sequence alignment (Durbin *et al.* (1998) *Biological sequence analysis – probabilistic models of proteins and nucleic acids.* Cambridge University Press, Cambridge, UK), functional search (Altschul *et al.* (1997) *Nucleic Acid Res.* 25:3389-3402) and PCR techniques (Sambrook and Russell (2001) *Molecular Cloning – a laboratory manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA). Some of these methods are described in Example 1 *supra*, and all are incorporated by reference.

[0019] In various embodiments, the isolated nucleic acid molecules span 0.1 kb, 0.5 kb, 1 kb, 2 kb, 3 kb, 4 kb or 5 kb starting at the ATG start codon for the coding region of the genes in question. The isolated nucleic acid molecules are referred to herein as promoters. Promoters correspond to the nucleic acid molecules whose function is to regulate the expression of a gene. A promoter generally comprises specific signaling sequences called boxes, arranged along the promoter sequence, such that its composition determines the temporal and spatial expression of a gene that is under its regulatory control. “Promoter” or “transcriptional initiation region” means a regulatory region of DNA usually comprising a TATA box capable of directing RNA polymerase II to initiate RNA synthesis at the appropriate transcription initiation site for a particular coding sequence. A promoter may additionally comprise other recognition sequences generally positioned upstream or 5' to the TATA box, referred to as upstream promoter elements, which influence the transcription initiation rate. It is recognized that, having identified the nucleotide sequences for the promoter regions disclosed herein, it is within the state of the art to isolate and identify further regulatory elements in the 5' untranslated region upstream from the particular promoter regions identified herein.

[0020] Thus the promoter regions disclosed herein are generally further defined by additional upstream regulatory elements such as those responsible for tissue and temporal expression of the coding sequence, enhancers and the like. In the same manner, the promoter elements, which enable expression in the desired tissue such as xylem and/or cambium, can be identified, isolated and used with other core promoters to confer cambium/xylem-preferred expression.

[0021] In the present invention, promoters that regulate the expression of genes specifically in the cambium and/or xylem were identified and isolated from *Populus* sp.

[0022] The SuSy gene encodes an isoform of sucrose synthase, an enzyme involved in the conversion of sucrose into UDP-glucose in the developing xylem. UDP-glucose is the building block of cellulose that is synthesized and deposited in the plant cell wall. The SuSy gene disclosed herein is preferentially expressed in the cambium/xylem of *Populus* sp, although low levels of expression can be observed in other tissues (FIG. 2).

[0023] The TUB gene encodes an isoform of alpha-tubulin, a structural globular protein involved in the formation of microtubules, which are part of the cytoskeleton. The TUB gene disclosed herein is preferentially expressed in the cambium and/or xylem of *Populus* sp, although low levels of expression can be observed in other tissues (FIG. 2).

[0024] The ARAB gene encodes an isoform of arabinogalactan protein, member of a large family of plant cell wall-associated glycoproteins of unknown function. The ARAB gene disclosed herein is preferentially expressed in the cambium/xylem of *Populus* sp, although low levels of expression can be observed in other tissues (FIG. 2).

[0025] The COMT gene encodes an isoform of caffeic acid 3-O-methyltransferase implicated in the methylation of both caffeic acid and 5-hydroxyferulic acid. These are intermediate compounds of lignin biosynthesis. The COMT gene disclosed herein is preferentially expressed in the cambium/xylem of *Populus* sp, although low levels of expression can be observed in other tissues (FIG. 3).

[0026] The CAD gene encodes an isoform of cinnamyl alcohol dehydrogenase, an enzyme that catalyzes the final step in the synthesis of monolignols, thereby converting the cinnamaldehydes to their corresponding alcohols. The CAD gene disclosed herein is preferentially expressed in the cambium/xylem of *Populus* sp, although low levels of expression can be observed in other tissues (FIG. 3).

[0027] The C4H gene encodes an isoform of cinnamate 4-hydroxylase, a member of the cytochrome P450 monooxygenase superfamily involved in the catalysis of the first oxidative reaction in the phenylpropanoid metabolism, namely the conversion of trans-cinnamic acid to *p*-coumaric acid. The C4H gene disclosed herein

is preferentially expressed in the cambium/xylem of *Populus* sp, although low levels of expression can be observed in other tissues (FIG. 3).

[0028] The CCR gene encodes an isoform of cinnamoyl CoA reductase, which catalyzes the conversion of cinnamoyl CoA esters to their corresponding cinnamaldehydes, i.e., the first specific step in the synthesis of lignin monomers. The CCR gene disclosed herein is preferentially expressed in the cambium/xylem of *Populus* sp, although low levels of expression can be observed in other tissues (FIG. 3).

[0029] The F5H gene encodes a cytochrome P450-dependent monooxygenase that catalyzes the hydroxylation of ferulic acid in a biosynthesis directed towards sinapic acid and syringyl lignin. The F5H gene disclosed herein is preferentially expressed in the cambium/xylem of *Populus* sp, although low levels of expression can be observed in other tissues (FIG. 3).

[0030] The SAD gene encodes a sinapyl alcohol dehydrogenase that mediates the reduction of sinapaldehyde into syringyl monolignols in angiosperms. The SAD gene disclosed herein is preferentially expressed in the cambium/xylem of *Populus* sp, although low levels of expression can be observed in other tissues (FIG. 3).

[0031] The UDP gene encodes the enzyme UDP-D-glucuronate carboxy-lyase involved in the breakdown of UDP-D-glucuronate into UDP-D-xylose and CO<sub>2</sub>. The UDP gene disclosed herein is preferentially expressed in the cambium/xylem of *Populus* sp, although low levels of expression can be observed in other tissues (FIG. 3).

[0032] The LTP gene encodes an isoform of lipid transfer protein, a member of a family thought to participate in cutin formation, embryogenesis, defense reactions against phytopathogens, symbiosis, and the adaptation of plants to various environmental conditions. The LTP gene disclosed herein is preferentially expressed in the cambium/ xylem of *Populus* sp, although low levels of expression can be observed in other tissues (FIG. 3).

[0033] The AG13 gene encodes an ag-13 protein of unknown function, whose expression has been associated with the ripening process in several plant species. The AG13 gene disclosed herein is preferentially expressed in the cambium/xylem of *Populus* sp, although low levels of expression can be observed in other tissues (FIG. 3).

[0034] The cambium/xylem-preferred promoter sequences of the present invention drive the expression of operably linked nucleotide sequences in a

cambium/xylem-preferred manner. EXAMPLE 4 illustrates the expression of the GUS reporter gene in the cambium/xylem vessels/fiber complex of *Arabidopsis thaliana* transformed with a construct containing the GUS reporter gene operably linked to two cambium/xylem-preferred promoters of the invention, i.e., the TUB (SEQ ID.: 2) and C4H (SEQ ID.: 6) promoters. EXAMPLE 4 also summarizes results showing expression of the GUS reporter gene in *Arabidopsis* plants transformed with constructs containing the GUS reporter gene operably linked to each one of the promoter sequences disclosed herein. Thus, the cambium/xylem-preferred promoter sequences disclosed herein can be used to express an operably linked sequence of interest in the cambium and/or in the xylem. Hence, the cambium/xylem-preferred promoters can be used to improve the wood quality of trees either by increasing the synthesis of cellulose or by decreasing the synthesis of lignin. "Decreasing lignin synthesis" means decreasing the total lignin content of woody trees by anywhere from 1-90%, preferably by about 80-90% relative to the lignin content in normal field grown plants. "Increasing cellulose synthesis" means increasing the total cellulose content of woody trees by 1-90%, preferably by about 80-90%, compared with normal field grown plants.

[0035] In addition, the cambium/xylem-preferred promoters can be used to inhibit the expression of genes involved in the metabolism of developing xylem. The inhibition of such genes decreases the concentration of lignin and/or changes the relationship between guaiacyl and syringyl, the building blocks of lignins. The monomeric composition of lignins is an important characteristic from the industrial point of view, because syringyl unit-rich lignins are more easily degraded during the pulping process, as they contain fewer strong 5-5' carbon bonds. Thus, the determination of the syringyl to guaiacyl (S/G) ratio is useful in evaluating wood quality for cellulose production and papermaking (Boudet et al., 1998). "Changing the relationship between syringyl and guaiacyl" refers to increasing the syringyl/guaiacyl ratio by 1-90%, preferably from about 80-90% compared with normal field grown plants.

[0036] Other nucleic acid molecules within the invention are variants and/or fragments of the cambium/xylem-preferred promoter sequences such as those that encode fragments, analogs or derivatives of native cambium/xylem-preferred promoter sequences disclosed herein. Such variants and/or fragments may be, e.g., naturally

occurring variants of native cambium/xylem-preferred promoter sequences, or non-naturally occurring variants of cambium/xylem-preferred promoter sequences. For example, the nucleotide sequence of such variants and/or fragments can include deletions, additions, and/or substitutions of one or more nucleotides as compared to the native cambium/xylem-preferred promoter sequences. Such variants and/or fragments may retain the biological activity and therefore drive, in a cambium/xylem-preferred manner, the expression of operably linked nucleotide sequences. Fragments of cambium/xylem-preferred promoter sequences comprise from about 10, to about 4000 nucleotides or up to the number of nucleotides in the full-length cambium/xylem-preferred promoter sequences disclosed herein as, such as the 700-3500 nucleotides of SEQ ID NOS.:1-12.

[0037] “Variants” is intended to include substantially similar sequences. Naturally and non-naturally occurring “variants” of cambium/xylem-preferred promoter sequences within the invention are nucleic acid molecules having at least 65% sequence identity with the native cambium/xylem-preferred promoter sequences disclosed herein, i.e., SEQ ID NOS: 1-12. “Variants” also include nucleic acids molecules that hybridize under stringent conditions, as defined herein, to the cambium/xylem-preferred promoter nucleic acid sequences of SEQ ID NOS.: 1-12 or the complement of the sequences of SEQ ID NOS.: 1-12. For example, such “variants” may be nucleic acid molecules that hybridize to the sequence of SEQ ID NOS.: 1-12 or the complement of the sequences of SEQ ID NOS: 1-12 under low stringency conditions, moderate stringency conditions, or high stringency conditions. Alternatively, such nucleic acids are those having a nucleotide sequence that is the complement of the full-length or portions of the sequences of SEQ ID NOS.: 1-12. Other variants of cambium/xylem-preferred promoter sequences within the invention are polynucleotides that share at least 65% sequence identity, preferably at least 80%, more preferably at least 90%, and most preferably at least 95%, to the sequences of SEQ ID NOS: 1-12 or the complement of the sequences of SEQ ID NOS: 1-12.

[0038] “Stringent conditions”, as used herein, refers to the parameters set forth supra.

[0039] For purposes of the present invention, sequence identity to any of the promoter sequences disclosed herein is preferably made using art known methodologies

such as the BLAST program, or any sequence alignment program that allows the alignment of identical nucleotides and verification of mismatches between non-identical nucleotides so that the percentage of identity of compared sequences could be estimated.

[0040] The cambium/xylem-preferred promoters of the invention may be used to express a gene of interest. For example, by using cambium/xylem-preferred promoters, the expression of native and/or non-native genes could be regulated in the cambium and/or xylem tissues of a plant, thus altering a plant's cellulose content, lignin content, pathogen or insect resistance, wood development, wood quality, and the like. The native and/or non-native genes include those encoding enzymes, transporters, cofactors, transcription factors and a number of other genes that would affect cellulose and/or lignin deposition in the plant or pathogen or insect resistance.

[0041] For the present invention, "genes of interest" include those involved in cellulose metabolism and lignin metabolism. It is recognized that any gene of interest can be operably linked to the promoter of the invention and expressed in plant cambium and/or xylem tissues.

[0042] The cambium/xylem-preferred promoters of the present invention, when operably linked to a gene of interest and stably incorporated into a plant genome, drive cambium and/or xylem-preferred expression of the said gene of interest. Cambium and/or xylem-preferred expression is intended to mean that expression of the gene of interest is most abundant in the cambium and/or in the xylem, although some level of expression of the gene of interest may occur in other plant tissues. Cambium encompasses any part of the cambial or procambial tissue in any organ of the plant, including but not being limited to the root, shoot, stem, wood, leaf, petiole, and the like. Xylem means any part of the xylem tissue, including but not being limited to the tracheids, tracheary elements, vessels, fuse fibers and pith. Some of the promoters disclosed herein may drive the expression of genes to the secondary xylem more prominently than to the primary xylem.

[0043] The constructs containing the cambium/xylem-preferred promoters disclosed in the present invention and an operably linked gene of interest may be provided in expression cassettes as depicted in the figures. Such expression cassettes comprise the cambium/xylem-preferred promoters of the present invention, or variants

or fragments thereof, operably linked to a gene of interest whose expression is directed to the cambium and/or xylem. Such an expression cassette may contain restriction sites for insertion of the gene of interest under the transcriptional control of the cambium/xylem-preferred promoters. The expression cassette may additionally contain a number of other nucleic acid sequences, including selectable marker genes; transcriptional and translational initiation sequences, and a plant transcriptional and translational termination sequence. The termination region may be native with the DNA sequence of interest or may be from the Ti-plasmid of *A. tumefaciens*, such as the octopine synthase and nopaline synthase termination regions (Gielen et al., EMBO J., 3:835-846 (1984), Depicker et al., Mol. and Appl. Genet., 1:561-573 (1982)).

[0044] Reporter genes or selectable marker genes may be included in the expression cassettes. Examples of suitable reporter genes known in the art can be found in, for example, Jefferson et al. (1991) in *Plant Molecular Biology Manual*, ed. Gelvin et al. (Kluwer Academic Publishers), pp. 1-33. Selectable marker genes for selection of transformed cells or tissues can include genes that confer herbicide resistance. Examples of suitable selectable marker genes include, but are not limited to, genes encoding resistance to sulfonamide (Guerineau et al. (1990) *Plant Mol. Biol.* 15:127-136), bromoxynil (Stalker et al. (1988) *Science* 242:419-423), glyphosate (Shaw et al. (1986) *Science* 233:478-481) and phosphinothricin (DeBlock et al. (1987) *EMBO J.* 6:2513-2518).

[0045] The expression cassettes of the present invention operably linked to a gene of interest are useful for the transformation of a variety of plants. Such plants, include, but are not limited to, *Eucalyptus* species (*E. alba*, *E. albens*, *E. amygdalina*, *E. aromaphloia*, *E. baileyana*, *E. balladoniensis*, *E. bicostata*, *E. botryoides*, *E. brachyandra*, *E. brassiana*, *E. brevistylis*, *E. brockwayi*, *E. camaldulensis*, *E. ceracea*, *E. cloeziana*, *E. coccifera*, *E. cordata*, *E. cornuta*, *E. corticosa*, *E. crebra*, *E. croajingolensis*, *E. curtisii*, *E. dalrympleana*, *E. deglupta*, *E. delegatensis*, *E. delicata*, *E. diversicolor*, *E. diversifolia*, *E. dives*, *E. dolichocarpa*, *E. dundastii*, *E. dunnii*, *E. elata*, *E. erythrocorys*, *E. erythrophloia*, *E. eudesmoides*, *E. falcata*, *E. gamophylla*, *E. glaucina*, *E. globulus*, *E. globulus* subsp. *bicostata*, *E. globulus* subsp. *globulus*, *E. gongylocarpa*, *E. grandis*, *E. grandis* x *urophylla*, *E. guilfoylei*, *E. gunnii*, *E. hallii*, *E. houseana*, *E. jacksonii*, *E. lansdowneana*, *E. latisinensis*, *E. leucophloia*, *E. leucoxylon*,

*E. lockyeri, E. lucasii, E. maidenii, E. marginata, E. megacarpa, E. melliodora, E. michaeliana, E. microcorys, E. microtheca, E. muelleriana, E. nitens, E. nitida, E. obliqua, E. obtusiflora, E. occidentalis, E. optima, E. ovata, E. pachyphylla, E. pauciflora, E. pellita, E. perriniana, E. petiolaris, E. pilularis, E. piperita, E. platyphylla, E. polyanthemos, E. populnea, E. preissiana, E. pseudoglobulus, E. pulchella, E. radiata, E. radiata subsp. radiata, E. regnans, E. risdonii, E. robertsonii, E. rodwayi, E. rubida, E. rubiginosa, E. saligna, E. salmonophloia, E. scoparia, E. sieberi, E. spathulata, E. staeri, E. stoatei, E. tenuipes, E. tenuiramis, E. tereticornis, E. tetragona, E. tetrodonta, E. tindaliae, E. torquata, E. umbra, E. urophylla, E. vernicosa, E. viminalis, E. wandoo, E. wetarensis, E. willisii, E. willisii subsp. *falciformis*, E. willisii subsp. *willisii*, E. *woodwardii*), *Populus* species (*P. alba*, *P. alba* x *P. grandidentata*, *P. alba* x *P. tremula*, *P. alba* x *P. tremula* var. *glandulosa*, *P. alba* x *P. tremuloides*, *P. balsamifera*, *P. balsamifera* subsp. *trichocarpa*, *P. balsamifera* subsp. *trichocarpa* x *P. deltoides*, *P. ciliata*, *P. deltoides*, *P. euphratica*, *P. euramericana*, *P. kitakamiensis*, *P. lasiocarpa*, *P. laurifolia*, *P. maximowiczii*, *P. maximowiczii* x *P. balsamifera* subsp. *trichocarpa*, *P. nigra*, *P. sieboldii* x *P. grandidentata*, *P. suaveolens*, *P. szechuanica*, *P. tomentosa*, *P. tremula*, *P. tremula* x *P. tremuloides*, *P. tremuloides*, *P. wilsonii*, *P. canadensis*, *P. yunnanensis*) and Conifers as, for example, loblolly pine (*Pinus taeda*), slash pine (*Pinus elliotii*), ponderosa pine (*Pinus ponderosa*), lodgepole pine (*Pinus contorta*), and Monterey pine (*Pinus radiata*); Douglas-fir (*Pseudotsuga menziesii*); Western hemlock (*Tsuga canadensis*); Sitka spruce (*Picea glauca*); redwood (*Sequoia sempervirens*); true firs such as silver fir (*Abies amabilis*) and balsam fir (*Abies balsamea*); and cedars such as Western red cedar (*Thuja plicata*) and Alaska yellow-cedar (*Chamaecyparis nootkatensis*).*

[0046] The expression cassettes may be stably incorporated into plant genomes by *Agrobacterium*-mediated transformation (Fraley *et al.* (1983) *Proc. Natl. Acad. Sci. USA*. 80:4803-4807) or by the biobalistics method (Klein *et al.* (1987) *Nature*. 327:70-73).

[0047] All technical terms used herein are terms commonly used in biochemistry, molecular biology and agriculture, and can be understood by one of ordinary skill in the art to which this invention belongs. Those technical terms can be found in: Molecular Cloning: A Laboratory Manual, 3rd ed., vol. 1-3, ed. Sambrook and

Russel, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 2001; Current Protocols in Molecular Biology, ed. Ausubel et al., Greene Publishing Associates and Wiley-Interscience, New York, 1988 (with periodic updates); Short Protocols in Molecular Biology: A Compendium of Methods from Current Protocols in Molecular Biology, 5<sup>th</sup> ed., vol. 1-2, ed. Ausubel et al., John Wiley & Sons, Inc., 2002; Genome Analysis: A Laboratory Manual, vol. 1-2, ed. Green et al., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1997. Methods involving plant biology techniques are described herein and are described in detail in methodology treatises such as Methods in Plant Molecular Biology: A Laboratory Course Manual, ed. Maliga et al., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1995. Various techniques using PCR are described, e.g., in Innis et al., PCR Protocols: A Guide to Methods and Applications, Academic Press, San Diego, 1990 and in Dieffenbach and Dveksler, PCR Primer: A Laboratory Manual, 2<sup>nd</sup> ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 2003. PCR-primer pairs can be derived from known sequences by using computer programs intended for that purpose (e.g., Primer, Version 0.5, 1991, Whitehead Institute for Biomedical Research, Cambridge, MA). Methods for chemical synthesis of nucleic acids are discussed, for example, in Beaucage and Caruthers (1981) Tetra. Lett. 22:1859-1862 and Matteucci and Caruthers (1981) J. Am. Chem. Soc. 103:3185.

[0048] The present invention is further illustrated by the following specific examples. The examples are provided for illustration only and are not to be construed as limiting the scope or content of the invention in any way.

## EXAMPLE 1

### Expression Profile of Genes Preferably Expressed in Cambium/Xylem

[0049] Expressed Sequence Tags (ESTs) from *Populus* sp. were clustered using the CAP3 program (Huang and Madan (1999) *Genome Res.* 9:868-877). Such ESTs were obtained from libraries representing the following tissues: apical shoot, bark, cambium, seed, xylem, leaf and root. The set of clusters thus generated was searched for those clusters composed of at least 90% of ESTs from libraries representing *Populus* cambium and xylem tissues. Twelve clusters were chosen based on their high and preferred level of expression in the cambium and/or in the xylem of *Populus*. A

BLASTX search against the non-redundant GenBank database was then performed with each one of the twelve clusters, and it was concluded that they represent expressed sequences from the following genes, sucrose synthase (SuSy), alpha-tubulin (TUB), arabinogalactan protein (ARAB), caffeic acid 3-O-methyltransferase (COMT), cinnamyl alcohol dehydrogenase (CAD), cinnamate 4-hydroxylase (C4H), cinnamoyl CoA reductase (CCR), ferulate-5-hydroxylase (F5H), sinapyl alcohol dehydrogenase (SAD), UDP-D-glucuronate carboxy-lyase (UDP), lipid transfer protein (LTP) and ag-13 (AG13). FIGs 2 and 3 show the expression profile in several tissues of *Populus* for each of the clusters representing the genes whose promoters are disclosed herein. The series of histograms in FIGs 2 and 3 ultimately depict the relative abundance of each gene in cDNA libraries representing the aforementioned tissues (apical shoot, bark; cambium, seed, xylem, leaf and root). Thus, the histograms compose a set of digital expression data which is an approximation of the relative level of expression for the twelve genes whose promoters are disclosed herein.

## EXAMPLE 2

### **Isolation of Promoter Sequences**

[0050] BLASTN was performed for each one of the twelve clusters against the genomic sequences from *Populus trichocarpa* made available by the Joint Genome Institute, US Department of Energy as part of the "Populus Genome Sequencing Project" (<http://genome.jgi-psf.org/poplar0/poplar0.info.html>). Selected nucleotide regions from each cluster corresponding to putative exons were used as driver sequences in the retrieval of genomic sequence reads comprising the transcription initiation region and adjacent upstream promoter sequences. These genomic reads were assembled using the PHRAP (Gordon *et al.* (1998) *Genome Res.* 8:195-202) program to obtain a contig encompassing approximately 700 to 3500 nucleotides of putative promoter region upstream from the transcription initiation point (+1 nucleotide, which corresponds to the beginning of the respective mRNA). These contigs contain the promoter regions for each of the genes encoding the mRNAs represented by the twelve clusters concluded to be preferably expressed in the cambium and/or in the xylem tissues of *Populus*. These twelve promoter regions correspond to sequences disclosed herein under SEQ ID NOS.: 1-12.

[0051] For isolation of specific promoter regions, pairs of gene-specific primers (usually 30 nt in length) were designed from the sequences of the promoter contigs described above to amplify by PCR a fragment of 700 to 3500 nucleotides from the promoter region of each one of the twelve genes whose promoter sequences are disclosed herein. The first round of PCR was performed on genomic DNA sample from *Populus deltoides* or *P. trichocarpa*, which was prepared from leaves using the cetyltrimethyl-ammonium bromide (CTAB) extraction method (Aldrich and Cullis (1993) *Plant Mol. Biol. Report.* 11:128-141). The primers were designed to amplify the region upstream of the coding sequence, i.e., the 5' untranslated region and promoter region of the chosen gene. The sequences of the primers used are given below for each promoter:

**sucrose synthase (SuSy)**

5' - GCCATAGCTCCTTAAGAGAACAGAAAGCAA - 3'	(SEQ ID NO: 13)
5' - CAATATAGAACATGAACAGCACTAGTTGC - 3'	(SEQ ID NO: 14)
5' - TCATGTCCTATCCAACGGCG - 3'	(SEQ ID NO: 15)

**alpha-tubulin (TUB)**

5' - CTCATTTCTCTCAAAGCTCAAAG - 3'	(SEQ ID NO: 16)
5' - GACAACTAGTCTAAAGTTAAACTTAGACC - 3'	(SEQ ID NO: 17)
5' - CCCTGGAGGTTGGGGTGAGT - 3'	(SEQ ID NO: 18)

**arabinogalactan protein (ARAB)**

5' - GCGTTCATCTACAAAACCCTCCTCC - 3'	(SEQ ID NO: 19)
5' - TTCATCCTTATTTTTGGGATA - 3'	(SEQ ID NO: 20)
5' - CAAAGGATCATGGAGTTGGA - 3'	(SEQ ID NO: 21)

**caffeic acid 3-O-methyltransferase (COMT)**

5' - TATACTAATATGACCTAATAACTTAGAAGTGTGG - 3'	(SEQ ID NO: 22)
5' - CATCTTGATCAAGATTGAATTC - 3'	(SEQ ID NO: 23)
5' - CATAATATCAAAACTTAAGC - 3'	(SEQ ID NO: 24)

**cinnamyl alcohol dehydrogenase (CAD)**

5' - TGAATTGATGACGTAGGAAACATGATAAACATG - 3'	(SEQ ID NO: 25)
5' - CATTTCCTGAAACAATGAGGCTAAGAG - 3'	(SEQ ID NO: 26)

**cinnamate 4-hydroxylase (C4H)**

5' - GACATGAGAAACTAACGTTGCTTGAATTG - 3'	(SEQ ID NO: 27)
5' - CATAATATTGGAACGGTTCTTCAGAAAG - 3'	(SEQ ID NO: 28)

**cinnamoyl CoA reductase (CCR)**

5' - GCGCTCGGGTTGTCACCATAGTTTC - 3'	(SEQ ID NO: 29)
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5' - CATGTTGTTATATTAGATAAAATGTA -3' (SEQ ID NO: 30)

**ferulate-5-hydroxylase (F5H)**

5' - TTCATCAAGCAATAATAATAAGGTGAGGC -3' (SEQ ID NO: 31)

5' - CATGGATGCAGATTTGTGTTGTG -3' (SEQ ID NO: 32)

5' - TTCAGTGAACATGCTGCCACAATGAC - 3' (SEQ ID NO: 33)

**sinapyl alcohol dehydrogenase (SAD)**

5' - AATCGAAACCGATCGATTGAACTGG -3' (SEQ ID NO: 34)

5' - CATGGTGCTTGCTTCAGATAG -3' (SEQ ID NO: 35).

**UDP-D-glucuronate carboxy-lyase (UDP)**

5' - GGAAATGTCAACACACTTGTGACACAC -3' (SEQ ID NO: 36)

5' - GACATTCTTGTCCAATTCTGAA -3' (SEQ ID NO: 37)

**lipid transfer protein (LTP)**

5' - GGAGCCTCCATATTCTGTATCTC -3' (SEQ ID NO: 38)

5' - CAAGACGATGAAATGAAGAACTGATAGC -3' (SEQ ID NO: 39).

**ag-13 (AG13)**

5' - GACATTCTTGACTTAATATGATGCT -3' (SEQ ID NO: 40)

5' - GAATTCGCATCCATGCGGTGAGTCG -3' (SEQ ID NO: 41)

[0052] PCR was performed using commercially available reagents and cycle parameters of 5 min at 94° C followed by 35 cycles of 94° C for 1 min, then a varying annealing temperature, as described infra for 1 min, then 72° C for 3 min. The annealing temperature (T) was adjusted for each primer pair and ranged from 50° C to 59° C. Finally, the samples were held at 72° C for 7 min, then at 4° C until further analysis. Ten  $\mu$ l of each of the resulting amplified DNA fragments were run on a 0.8% agarose gel, purified using the GFX Gel Purification Kit (Amersham), subcloned into pGEM-T-Easy vector (Promega) and then into EcoRI and BglII sites of the pAPROM-ATG vector. Final sequences were determined on the resulting plasmids. Figure 1 schematically illustrates the expression cassette pAPROM-ATG comprising the GUS gene operably linked to a promoter disclosed herein. Figure 4 schematically illustrates the plasmid vector comprising a gene of interest operably linked to a promoter of the invention.

### EXAMPLE 3

#### Transformation of *Arabidopsis* Plants

[0053] *Arabidopsis thaliana* Columbia plants were transformed using an *Agrobacterium tumefaciens* mediated transformation protocol (Bechtold *et al.*, (1993) *C. R. Acad. Sci. Paris* 316:1194-1199; Bent *et al.*, (1986) *Mol. Gen. Genet.* 204:383-396) with individual constructs containing any one of the promoters of the invention operably linked to a gene of interest. The constructs also contained the selectable marker gene *Bar* that confers resistance to herbicidal phosphinothricin analogs like ammonium gluphosinate (Thompson *et al.* (1987) *EMBO J.* 9:2519-2523). In this example, the gene of interest operably linked to the cambium/xylem-preferred promoters of the invention is the reporter gene *Gus* encoding the enzyme beta-glucuronidase (GUS) (Jefferson (1987) *Plant Mol. Biol. Rep.* 5: 387-405) that facilitates visual inspection of the desirable phenotype, i.e., expression of GUS in a cambium/xylem-preferred manner.

[0054] Seeds of *Arabidopsis thaliana* ecotype Columbia were sown in pots containing vermiculite. Plants were grown under a 16/8 hours dark/light regime at 22 °C. After 4-5 weeks, plants were transformed with the *Agrobacterium tumefaciens* strain GV3101 in accordance with Bent *et al.*, (1986) *Mol. Gen. Genet.* 204:383-396; which harbors the plasmid vector comprising the gene of interest operably linked to each one of the promoters of the invention.

[0055] For plant transformation, 1 liter of LB medium containing rifampicin, gentamycin and kanamycin was inoculated with an aliquot of overnight starter *Agrobacterium* culture. The culture was then grown overnight at 28 °C in a rotary shaker, until OD600 is  $\geq 8.0$ . The *Agrobacterium* was precipitated by centrifugation and the bacterial pellet was resuspended in ~300 ml of 5% sucrose and 0.03% Silwet L-77. This *Agrobacterium* suspension was sprayed onto the plants. The pots were placed in a tray which was covered with plastic wrap to maintain humidity and the plants were grown at the above regime, in order to mature and to set seeds.

[0056] Seeds were harvested and surface sterilized in a solution containing 50% bleach and 0.02% Triton X-100 for 7 minutes. Seeds were then rinsed 3 times in sterile distilled water and plated out in MS medium containing 6 mg/l of Finale as a selection agent. After 5 to 7 days, transformants were visible as green plants. Transformed

plants were transferred onto new selection plates and after 6-10 days were transferred to pots containing vermiculite and grown under conditions of 16 hours light/ 8 hours dark at 22 °C.

#### EXAMPLE 4

##### **GUS expression assay in *Arabidopsis* plants**

[0057] Inflorescence stems of the transformed plants described in EXAMPLE 3 were cut and histologically stained for GUS activity. Subsequent cuttings induced the formation of secondary xylem at the basis of plants that could also be histologically stained for GUS activity.

[0058] In figures 5A and 5B, activity of beta-glucuronidase in flowering stems of transgenic *Arabidopsis* plants is shown. These transgenic *Arabidopsis* plants were transformed with a construct containing the gene *Gus* operably linked to cambium/xylem-preferred promoters of the invention, namely TUB (SEQ ID.: 2) (A) and C4H (SEQ ID.: 6) (B). Darker bands along the longitudinal axis of the stem (arrowheads) represent primary vascular bundles stained blue after the chromogenic assay, indicating the functionality and tissue-specificity of the respective promoter in each transgenic line.

[0059] The table below summarizes GUS assay data obtained through the analysis of inflorescence stem cuttings of *Arabidopsis thaliana* plants transformed with expression constructs according to EXAMPLE 2 comprising the *Gus* gene under the control of promoter sequences disclosed herein. For all promoters tested, vascular GUS expression pattern was observed. In some cases, GUS activity was markedly high in specific vascular cell types such as vessel elements, as for example in plants transformed with constructs comprising the LTP (SEQ ID.: 11), C4H (SEQ ID.: 6) or TUB (SEQ ID.: 2) promoters. In other cases, a vascular pattern was observed but no specific cell type therein could be pinpointed as the main GUS expression site.

Promoter	No. of events analyzed	Total of GUS-positive events	Expression pattern		
			Vessel elements only	Vessel elements + other vascular cell types	Non-vascular cell types
SUSY	92	21	1	17	3
LTP	75	38	19	14	5
C4H	89	43	14	28	1
TUB	78	20	9	10	1
COMT	72	24	2	15	7
CAD	79	37	8	16	13
SAD	75	30	4	13	13
UDP	72	20	4	14	2
CCR	74	22	4	14	4

[0060] Other aspects of the invention will be clear to the skilled artisan, and need not be reiterated here.

[0061] The terms and expression which have been employed are used as terms of description and not of limitation, and there is no intention in the use of such terms and expression of excluding any equivalents of the features shown and described or portions thereof, it being recognized that various modifications are possible with the scope of the invention.